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PATENT
Attorney Docket No.: UCSD1330-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Palsson, et al.	Art Unit:	1632
Serial No.:	10/087,441	Examiner	Unassigned
Filed:	March 1, 2002		
Title:	MODELS AND METHODS FOR DETERMINING SYSTEMIC PROPERTIES OF REGULATED REACTION NETWORKS		

BOX AMENDMENT (NO FEE)
Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL SHEET

Sir:

Transmitted herewith for the above-identified application please find one (1) Preliminary Amendment.

Applicant does not believe there any fees in connection with this filing although the Commissioner is hereby authorized to charge any additional fees required by this filing, or credit any overpayment, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: May 20, 2002

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CERTIFICATION UNDER 37 CFR §1.8

I hereby certify that the documents referred to as enclosed herein are being deposited with the United States Postal Service as first class mail on May 20, 2002, in an envelope addressed to: BOX AMENDMENT (NO FEE), Commissioner for Patents, Washington, D.C. 20231

Lisa Corrales

Gray Cary\GT\6291993.1
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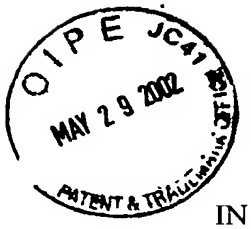

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Art Unit: 1632
Examiner: To be assigned

BOX AMENDMENT (NO FEE)
Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

In connection with the above-identified patent application, entry of the Preliminary Amendment is respectfully requested.

CERTIFICATION UNDER 37 CFR §1.8
I hereby certify that the documents referred to as enclosed herein are being deposited with the United States Postal Service as first class mail on this date, May 20, 2002, in an envelope addressed to:BOX AMENDMENT (NO FEE) Commissioner for Patents, Washington, D C. 20231.
Lisa Corrales *(Name of Person Mailing Paper)*
(Signature) May 20, 2002 *(Date)*

Gray Cary\GT\6294792.1
101668-66

I. AMENDMENTS

A. In the Specification

Please enter rewritten paragraph 11 on page 3 to read as follows:

Figure 3 shows a schematic drawing of a regulatory network associated with a reaction in a metabolic network. Integration of a stoichiometric model and a logical model is achieved through regulatory restraints (logic values of reaction processes) which are used to refine appropriate reaction constraints in the model. If rxnLogic = 1 then use Activity constraints; If rxnLogic = 0 then use Inactivity constraints. Activity constraints set for rxn_{stoich}: (lower bound = 0, upper bound = INF or #); Inactivity constraints for rxn_{stoich}: (lower bound = 0, upper bound = 0). Logic functions: a_1 = (activator/inhibtor) • TF; $a_2 = 1$; c_1 = TF* • pr_1 • *gene1* • *gene2;* $c_2 = pr_3$ • *gene3;* $1_1 = M_{gene1}$; $1_2 = M_{gene2}$; $1_3 = M_{gene3}$; $p1 = P_{gene1}$; • P_{gene2} • P_{gene3}; rsn_{Logic} = Protein • Cofactor • $Substrate_1$ • $Substrate_2$. Time delays can be specified for the switching of each memorization variable after a triggering change in the associated function.

Please enter rewritten paragraph 15 on page 4 to read as follows:

Figure 7 shows a schematic drawing of a simplified core metabolic network. Table 4 provides the stoichiometry of the 20 metabolic reactions included in the network.

Please enter rewritten paragraph 16 on page 4 to read as follows:

Figure 8 shows, in Panel A, a simulation of aerobic growth of *E. coli* on acetate with glucose reutilization; and in Panel B, *in silico* arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Panel A shows three time plots showing experimental data (closed squares or triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-alone metabolic model (dashed lines). Table 5 provides parameters used to generate the plots in Panel A.

Please enter rewritten paragraph 17 on page 4 to read as follows:

Figure 9 shows, in Panel A, a simulation of anaerobic growth of E. coli on glucose; and in Panel B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 6 provides the parameters used to generate the plots in Panel A.

Please enter rewritten paragraph 18 on page 4 to read as follows:

Figure 10 shows, in Panel A, a simulation of aerobic growth of E. coli on glucose and lactose; and in Panel B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 7 provides the parameters used to generate the plots of Panel A. Panel A shows time plots showing experimental data (triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (thick solid lines), the stand-alone metabolic model (dashed lines), and the kinetic model described in Kremling (*Metabolic Eng.* 3:362-379 (2001)) (thin solid line).

Please enter rewritten paragraph 139 on page 48 to read as follows:

A skeleton of the biochemical reaction network of core metabolism was formulated, including 20 reactions, 7 of which are regulated as shown in Figure 7. This network provided a simplified representation of core metabolic processes including glycolysis, the pentose phosphate pathway, TCA cycle, fermentation pathways, amino acid biosynthesis and cell growth, along with corresponding regulation pathways including catabolite repression, aerobic/anaerobic regulation, amino acid biosynthesis regulation and carbon storage regulation. The skeleton biochemical reaction network was represented as a skeleton combined regulatory/metabolic model where reactions were represented as linear equations of reactants and stoichiometric coefficients and regulation was represented by regulatory logic statements as shown in Table 4. As shown in Figure 7 and Table 4, four regulatory proteins (Rpo2, RPc1,RPh and RPb) regulated 7 of the 20 reactions in the skeletal network and model.

Please enter rewritten paragraph 156 on page 53 to read as follows:

E. coli has been observed *in vivo* to secrete acetate when grown aerobically on glucose in batch cultures; when glucose is depleted from the environment, the acetate is then reutilized as a substrate. Using the combined regulatory/metabolic and stand-alone metabolic models, activity of an aerobic batch culture of *E. coli* on glucose minimal medium was simulated. Panel A of Figure 8 shows three time plots showing experimental data (closed squares) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-alone metabolic model (dashed lines). In the acetate plot, the regulatory/metabolic model predictions differed from that of the stand-alone metabolic model, as shown. Table 5 provides the parameters required to generate the time plots where parameters were estimated or obtained from Varma and Palsson Appl. Env. Micro. 60:3724-3731 (1994). The major difference between the combined regulatory/metabolic and metabolic stand-alone simulations is in the delayed reaction of the system to depletion of glucose in the growth medium. The stand-alone metabolic network is unable to account for the delays associated with protein synthesis.

Please enter rewritten paragraph 159 on page 54 to read as follows:

The *in silico* models were used to simulate anaerobic growth on glucose, the results of which are shown in Figure 9 which was generated using the parameters provided in Table 6. Under these conditions, the stand-alone metabolic model made similar predictions as the combined regulatory/metabolic model, with a notable exception: the combined regulatory/metabolic model was able to make predictions about the use of a particular isozyme. For example, both models require fumarase activity as part of the optimal flux distribution; however, of the two models only the combined regulatory/metabolic model was able to specifically determine that the *fumB* gene product [which as being] is expressed under anaerobic conditions.

Please enter rewritten paragraph 160 on page 55 to read as follows:

Aerobic growth of *E. coli* on glucose and lactose was simulated using the *in silico* models and compared to *in vivo* observations from mixed batch cultures and to results reported for a kinetic model as described in Kremling et al., Metabolic Eng. 3:362-379 (2001). Overall, the combined regulatory/metabolic model predictions were in good agreement with the *in vivo* observations, comparable with the predictions made by the Kremling model, and better than the predictions of the stand-alone metabolic model as shown in Figure 10 which was generated using the parameters provided in Table 7. The deficiencies in the ability of the stand-alone metabolic model to accurately predict the results of this experiment is most likely due to the concurrent uptake of glucose and lactose, resulting in much more rapid depletion of the substrates and a higher growth rate. Interestingly, because of the larger flux of carbon source uptake, the stand-alone metabolic model predicted that *E. coli* growth should be oxygen-, rather than carbon-limited in this case. Accordingly, the secretion of acetate and formate was predicted by the stand-alone metabolic model. In contrast, the combined regulatory/metabolic model predicted that no secretion will occur under these conditions.

Please enter rewritten paragraph 161 on page 55 to read as follows:

The *in silico* arrays for the simulation (Figure 10B) showed one shift in gene expression, occurring just under five hours. The up-regulation of the lactose uptake and degradation machinery, together with key enzymes in galactose metabolism, enables the system to use lactose as a carbon source once the glucose in the medium has been depleted.

Please add the following:

After the second page of Table 3 on page 62, please add the following tables:

--

Table 4 provides the stoichiometry of the 20 metabolic reactions include in the network of Figure 7.

REACTION	NAME	REGULATION
Metabolic Reactions		
-1 A –1 ATP + 1 B	R1	
-1 B +2 ATP +2 NADH +1 C	R2a	IF NOT (RPb)
-1 C –2 ATP –2 NADH +1 B	R2b	
-1 B +1 F	R3	
-1 C +1 G	R4	
-1 G +0.8 C +2 NADH	R5a	IF NOT (RPo2)
-1 G +0.8 C +2 NADH	R5b	IF RPo2
-1 C +2 ATP +3 D	R6	
-1 C –4 NADH +3 E	R7	IF NOT (RPb)
-1G –1 ATP – 2 NADH +1 H	R8a	IF NOT (RPh)
+1 G +1 ATP +2 NADH –1 H	R8b	
-1 NADH –1.02 +1 ATP	Rres	IF NOT (RPo2)
Transport Processes		
-1 Carbon1 +1 A	Tc1	
-1 Carbon2 +1 A	Tc2	IF NOT (RPc1)
-1 Fext +1 F	Tf	
-1 D +1 Dext	Td	
-1 E +1 Eext	Te	
-1 Hext +1 H	Th	
-1 Oxygen +1 O2	To2	
Maintenance/Growth Processes		
-1 C –1 F –1 H –10 ATP +1 Biomass	Growth	
Regulatory Proteins		
	RPo2	IF NOT (Oxygen)
	RPc1	IF Carbon1
	RPh	IF Th
	RPb	IF R2b

Table 5 provides parameters used to generate the plots in Figure 8A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.003	Estimated
$[Glucose]_0$ (mM)	10.4	Estimated
$[Acetate]_0$ (mM)	0.3	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.4	Estimated
Biomass scaling factor	1.3	Varma and Palsson, 1994
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	10.5	Varma and Palsson, 1994
Acetate	2.5	Estimated
Oxygen	15.0	Varma and Palsson, 1994

Table 6 provides the parameters used to generate figure 9A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.002	Estimated
$[Glucose]_0$ (mM)	10.5	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.4	Estimated
Biomass scaling factor	1.3	Varma and Palsson, 1994
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	18.5	Varma and Palsson, 1994
Oxygen	0.0	Varma and Palsson, 1994

Table 7 provides the parameters used to generate the plots of Panel 10A.

Parameters		
Initial Conditions		
$[Biomass]_0$ (g/L)	0.011	Estimated
$[Glucose]_0$ (mM)	1.6	Estimated
$[Lactose]_0$ (mM)	5.8	Estimated
Strain-specific parameters		
Protein synthesis/degradation delay (hrs)	0.5	Estimated
Unconstraining [*lacZ*] (mmol/gDW)	0.0274	Estimated
Constraining [*lacZ*] (mmol/gDW)	0.0015	Estimated
*Uptake rate constraints (mM/(gDCW*hr))*		
Glucose	6.5	Estimated
Acetate	3.0	Estimated
Oxygen	15.0	Estimated

II. REMARKS

In connection with the above-identified patent application, entry of the Preliminary Amendment is respectfully requested. The amendments to the specification provided herein incorporate information that was included in the drawings as filed, but is being incorporated into the specification with this amendment because it is being deleted from the drawings with the replacement drawings filed May 20, 2002 in the Response to Notice to File Missing Parts. No new matter has been added with the amendments provided herein.

In view of the amendments and the above remarks, it is submitted that the claims are in condition for allowance and a notice to that effect is respectfully requested. The Examiner is invited to contact Applicants' undersigned representative if there are any questions relating to this application.

Please charge any additional fees, or make any credits, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: May 20, 2002

Lisa Haile, J.D., Ph.D.
Registration No. 38,347
Telephone: (858) 677-1456
Facsimile: (858) 677-1465
Respectfully submitted,

CUSTOMER NUMBER 28213
GRAY CARY WARE & FREIDENRICH LLP
4365 Executive Drive, Suite 1100
San Diego, California 92121-2133

Enclosures: Exhibit A

In re Application of:
Palsson et al.
Application No.: 10/087,441
Filed: March 1, 2002

PATENT
Attorney Docket No.: UCSD1330-2

EXHIBIT A

MARKED-UP COPY OF THE SPECIFICATION AND THE CLAIMS SHOWING THE AMENDMENTS

A. In the Specification

Please amend paragraph 11 on page 3 as follows:

Figure 3 shows a schematic drawing of a regulatory network associated with a reaction in a metabolic network. Integration of a stoichiometric model and a logical model is achieved through regulatory restraints (logic values of reaction processes) which are used to refine appropriate reaction constraints in the model. If rxnLogic = 1 then use Activity constraints; If rxnLogic = 0 then use Inactivity constraints. Activity constraints set for rxn_{stoich}: (lower bound = 0, upper bound = INF or #); Inactivity constraints for rxn_{stoich}: (lower bound = 0, upper bound = 0). Logic functions: a_1 = (activator/inhibtor) • TF; $a_2 = 1$; $c_1 = TF^* \bullet pr_1 \bullet$ *gene1* • *gene2;* $c_2 = pr_3 \bullet$ *gene3;* $1_1 = M_{gene1}$; $1_2 = M_{gene2}$; $1_3 = M_{gene3}$; $p1 = P_{gene1}$; • $P_{gene2} \bullet P_{gene3}$; rsn_{Logic} = Protein • Cofactor • $Substrate_1$ • $Substrate_2$. Time delays can be specified for the switching of each memorization variable after a triggering change in the associated function.

Please amend paragraph 15 on page 4 as follows:

Figure 7 shows a schematic drawing of a simplified core metabolic network[, together with a table containing] Table 4 provides the stoichiometry of the 20 metabolic reactions included in the network.

Please amend paragraph 16 on page 4 as follows:

Figure 8 shows, in Panel A, a simulation of aerobic growth of *E. coli* on acetate with glucose reutilization; [in Panel B, a table of parameters used to generate the plots in Panel A;] and in Panel [C] B, *in silico* arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Panel A shows three time plots showing experimental data (closed squares or triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-

alone metabolic model (dashed lines). Table 5 provides parameters used to generate the plots in Panel A.

Please amend paragraph 17 on page 4 as follows:

Figure 9 shows, in Panel A, a simulation of anaerobic growth of E. coli on glucose; [in Panel B, a table of parameters used to generate the plots in Panel A;] and in Panel [C] B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 6 provides the parameters used to generate the plots in Panel A.

Please amend page paragraph 18 on page 4 as follows:

Figure 10 shows, in Panel A, a simulation of aerobic growth of E. coli on glucose and lactose;[in Panel B, a table of parameters used to generate the plots in Panel A;] and in Panel [C] B, in silico arrays showing the up- or down-regulation of selected genes, or activity of regulatory proteins, in the regulatory network. Table 7 provides the parameters used to generate the plots of Panel A. Panel A shows time plots showing experimental data (triangles) and the corresponding simulations performed using the combined regulatory/metabolic model (thick solid lines), the stand-alone metabolic model (dashed lines), and the kinetic model described in Kremling (*Metabolic Eng.* 3:362-379 (2001)) (thin solid line).

Please amend paragraph 139 on page 48 as follows:

A skeleton of the biochemical reaction network of core metabolism was formulated, including 20 reactions, 7 of which are regulated as shown in [the upper panel of] Figure 7. This network provided a simplified representation of core metabolic processes including glycolysis, the pentose phosphate pathway, TCA cycle, fermentation pathways, amino acid biosynthesis and cell growth, along with corresponding regulation pathways including catabolite repression, aerobic/anaerobic regulation, amino acid biosynthesis regulation and carbon storage regulation. The skeleton biochemical reaction network was represented as a skeleton combined

regulatory/metabolic model where reactions were represented as linear equations of reactants and stoichiometric coefficients and regulation was represented by regulatory logic statements as shown in [the lower panel of Figure 7] Table 4. As shown in Figure 7 and Table 4, four regulatory proteins (Rpo2, RPc1,RPh and RPb) regulated 7 of the 20 reactions in the skeletal network and model.

Please amend paragraph 156 on page 53 as follows:

E. coli has been observed *in vivo* to secrete acetate when grown aerobically on glucose in batch cultures; when glucose is depleted from the environment, the acetate is then reutilized as a substrate. Using the combined regulatory/metabolic and stand-alone metabolic models, activity of an aerobic batch culture of *E. coli* on glucose minimal medium was simulated. Panel A of Figure 8 shows three time plots showing experimental data (closed squares) and the corresponding simulations performed using the combined regulatory/metabolic model (solid lines) as well as the stand-alone metabolic model (dashed lines). In the acetate plot, the regulatory/metabolic model predictions differed from that of the stand-alone metabolic model, as shown. [Panel B of Figure 8 shows a table containing] Table 5 provides the parameters required to generate the time plots where parameters were estimated or obtained from Varma and Palsson Appl. Env. Micro. 60:3724-3731 (1994). The major difference between the combined regulatory/metabolic and metabolic stand-alone simulations is in the delayed reaction of the system to depletion of glucose in the growth medium. The stand-alone metabolic network is unable to account for the delays associated with protein synthesis.

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For example, both models require fumarase activity as part of the optimal flux distribution; however, of the two models only the combined regulatory/metabolic model was able to specifically determine that the *fumB* gene product [which as being] is expressed under anaerobic conditions.

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Aerobic growth of *E. coli* on glucose and lactose was simulated using the *in silico* models and compared to *in vivo* observations from mixed batch cultures and to results reported for a kinetic model as described in Kremling et al., Metabolic Eng. 3:362-379 (2001). Overall, the combined regulatory/metabolic model predictions were in good agreement with the *in vivo* observations, comparable with the predictions made by the Kremling model, and better than the predictions of the stand-alone metabolic model as shown in Figure 10 which was generated using the parameters provided in Table 7. The deficiencies in the ability of the stand-alone metabolic model to accurately predict the results of this experiment is most likely due to the concurrent uptake of glucose and lactose, resulting in much more rapid depletion of the substrates and a higher growth rate. Interestingly, because of the larger flux of carbon source uptake, the stand-alone metabolic model predicted that *E. coli* growth should be oxygen-, rather than carbon-limited in this case. Accordingly, the secretion of acetate and formate was predicted by the stand-alone metabolic model. In contrast, the combined regulatory/metabolic model predicted that no secretion will occur under these conditions.

Please amend paragraph 161 on page 55 as follows:

The *in silico* arrays for the simulation (Figure [10C]10B) showed one shift in gene expression, occurring just under five hours. The up-regulation of the lactose uptake and degradation machinery, together with key enzymes in galactose metabolism, enables the system to use lactose as a carbon source once the glucose in the medium has been depleted.